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Immunoglobulin class and subclass restriction of autoimmune responses in secondary syphilis

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SUMMARY

The immunoglobulin (Ig) class and IgG subclasses of autoantibodies to commercial VDRL antigen, creatine kinase (CK), and fibronectin (Fn) in the sera of patients with various stages of syphilis were quantified using solid-phase radioimmunoassays (RIA) and ELISA. Sera from patients with active secondary syphilis, initially positive for anti-Fn and anti-CK autoantibodies by RIA, were re-evaluated by ELISA using monoclonal antibodies (MoAb) for detection of human Ig class and subclass responses. Results of these assays revealed that anti-Fn and anti-CK responses were not only IgG in nature, but dramatically skewed to IgG1 and IgG3 subclasses. While the restricted, co-expression of these isotypes seemingly paralleled anti-treponemal activity, inverse relationships actually existed between the subclass responses to Fn and those to *Treponema pallidum*. In contrast, anti-VDRL were predominantly IgM in 17 of 22 patients. Of those sera exhibiting detectable anti-VDRL IgG activity, responses appeared to be restricted to IgG1. These results suggest that different control mechanisms may be responsible for regulation of the various autoantibody responses expressed during syphilitic infection.

Keywords IgG subclasses autoimmune responses secondary syphilis

INTRODUCTION

Several recent studies, in both the human host and established animal models, suggest that the autoimmune phenomena associated with syphilitic infection is not restricted solely to anti-cardiolipin responses. Antibodies to fibronectin (Fn) (Fitzgerald *et al.*, 1984; Fitzgerald & Repesh, 1985; Baughn *et al.*, 1986; Baughn, *et al.*, 1987), to laminin and collagen (Fitzgerald *et al.*, 1984), and to muscle creatine kinase (Casavant, Wicher & Wicher, 1978; Strugnell *et al.*, 1986; Baughn *et al.*, 1987) have also been described suggesting that dynamic immunopathologic processes may be triggered by *Treponema pallidum* in susceptible hosts. While autoantibodies appear to contribute to both the formation of immune complexes (Baughn, 1987) and lesion histopathology (Jorizzo *et al.*, 1986, 1988), little is known about the mechanism(s) leading to their induction.

The goal of the present study was to quantitatively assess autoimmune responses with respect to isotype profile and then determine the extent of co-ordination between these class and subclass responses and those invoked against *T. pallidum*.

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MATERIALS AND METHODS

Human sera

All sera used in the present study had been previously characterized with respect to levels of immune complexes and antibody (Ig class and subclass) responses to outer membrane proteins of *T. pallidum* (Baughn *et al.*, 1986; Baughn *et al.*, 1988). Twenty-two sera were from patients with secondary syphilis, six from patients with primary syphilis, 11 from individuals who had been treated 6 months to 12 years previously for secondary syphilis, and 15 healthy adults with no history or serologic evidence of *T. pallidum* infection. Serologic tests for syphilis [VDRL slide test, FTA-ABS and the microhaemagglutination-*T. pallidum* (MHATP)] were performed in accordance with standard procedures (Larsen *et al.*, 1984).

Antigen preparations

Type IV collagen, rabbit muscle CK (RM-CK), beef heart CK (BH-CK) and laminin were obtained from Sigma Chemical Co. (St Louis, MO). The VDRL antigen was obtained from DIFCO Laboratories (Detroit, MI). Bovine and human Fn and human serum albumin (HSA) were obtained from Calbiochem-Behring (La Jolla, CA). Cell-binding fragments (CBF) or domains of human and bovine Fr were purified as previously described (Baughn, 1986). Polymerized human serum albumin (HSA) was

obtained according to previously described methods (Onica, Lenkei & Ghetie, 1978). Purified human MM isoenzyme CK (MM-CK) from skeletal muscle was a gift from Dr M. Benjamin Perryman (Baylor College of Medicine, Houston, TX).

Immunologic reagents

Unconjugated and alkaline phosphatase-conjugated second antibodies against human and mouse IgG and IgM were obtained from Microbiological Associates (Bethesda, MD), Cappel/Worthington Biochemicals, Cooper Biomedical (Malvern, PA), ICN ImmunoBiologicals, (Lisle, IL), or Tago (Burlingame, CA). Mouse MoAb to human IgG (GG-5) and IgM (MB-11) were obtained from ICN ImmunoBiologicals. MoAb against the human IgG subclasses were obtained from Unipath (Bedford, UK) and from the World Health Organization (WHO) Collaborating Center for Human Immunoglobulin Subclasses, CDC (Atlanta, GA). IgG subclass reference preparations were obtained from the WHO/International Union of Immunologic Societies Immunoglobulin Subcommittee, (Berne, Switzerland). Immunoglobulin fractions and MoAb, following chromatography on MAPS II (Bio-Rad Laboratories, Richmond, CA) to remove carrier bovine serum albumin (BSA), were labelled with ^{125}I-Na as previously described (Baughn *et al.*, 1988).

VDRL ELISA

The ELISA method for the detection of VDRL antibodies (Gharavi *et al.*, 1986; Pedersen, Orum & Mourtisen 1987; Harris *et al.*, 1988) was modified as follows. One-half of the wells of flat-bottomed, polystyrene Immunolon 2 plates (Dynatech Laboratories, Alexandria, VA) were coated with 50 μl of a 1:4 solution of phosphate-buffered saline (PBS) in absolute ethanol (background controls/non-specific binding) and the other half coated with 50 μl of VDRL antigen diluted 1:4 in ethanol (test wells). Plates were allowed to dry overnight at 37°C and subsequently washed three times with PBS, blocked for 2 h with 10% fetal calf serum (FCS) in PBS, and rewashed. One-hundred-μl aliquots of sera at appropriate dilutions in PBS with 10% FCS were then added to triplicate wells, and the plates incubated for 90 min at room temperature. Two normal reference sera, two syphilitic reference sera and an anti-CL standard serum were included in each assay. Plates were then washed three times and 100-μl aliquots of alkaline phosphatase-conjugated goat IgG against human IgM or IgG in PBS with 10% FCS were added to all wells. The plates were incubated for 90 min, washed three times and 100 μl of phosphatase substrate (Sigma 104, Sigma) in diethanolamine buffer, pH 9·8, was added to each well. Reactions were stopped at the end of a 45-min incubation period at room temperature by adding 100 μl of 3 M NaOH to each well. Absorbance was determined in an automated spectrophotometer at 405 nm (Dynatech). Net differences in optical absorbance were calculated by subtracting the optical absorbance of wells coated with PBS alone (no antigen). Positive results were defined as $> 5{\cdot}0$ s.d. above the mean of the values of the 15 normal control sera, which yielded consistently negative results on five separate occasions. Using standard curves constructed with the affinity-purified preparation of the anti-CL reference standard, OD values were converted to μg/ml.

Microassays for the detection of autoantibodies to host proteins

Radioimmunoassays (RIA), carried out as previously described (Baughn *et al.*, 1986, 1987), were used to screen sera at a 1:100 dilution for autoantibodies. Polyvinyl microtitre wells for these assays were coated with 100 μl of antigen in 0·1 M carbonate buffer (pH 9·6). This volume contained 40 μg of CK, 40 μg of type IV collagen, 2 μg of Fn-CBF, 2 μg of laminin, or 40 μg of polymerized HSA per ml. Certain sera in each RIA were subjected to depletion analysis (Hussain *et al.*, 1981) using both an antigen-specific sorbent and non-relevant antigen sorbent (BSA-Sepharose). Preparations before and after depletion were assayed by quantitative immunodiffusion in order to convert ct/min to μg/ml.

Sera yielding positive results in RIA were retested by ELISA in an attempt to quantify isotype specificity. For these assays, Immunolon 2 plates were used and antigen-coated plates initially blocked for 1 h at room temperature with 1% BSA in PBS. Control and test sera were diluted in PBS containing 0·5% BSA, and 0·1% bovine gamma globulin (BGG) and 100 μl of twofold dilutions (starting at 1/25) were added to triplicate wells and incubated for 2 h at room temperature. Using the recommendation of Jefferis *et al.* (1985), mixtures of the following mouse MoAb at appropriate dilutions were used for each of the subclass determinations: clones NL16, 2B6 and 8c/6-39 for IgG1; HP6002 (GOM1) and HP6014 for IgG2; HP6050 and ZG4 for IgG3; and RJ4 and 186 for IgG4. Dilutions of these MoAb mixtures were made in PBS containing 0·5% BSA and 0·1% BGG. A cocktail containing all of the MoAb (designated *pan*-MoAb) was used, in addition to GG-5 and a polyclonal anti-IgG, to assess total IgG responses. Antibody binding was then detected with alkaline phosphatase-conjugated goat anti-mouse (human adsorbed) IgG (Tago). After washing the wells five times with PBS, 100 μl of phosphatase substrate in diethanolamine buffer were added to each well. Subsequent steps in these assays were identical to those described above for the VDRL ELISA. Sets of myeloma proteins were included in each assay to ensure MoAb reactivity, negate cross-reactivity, and generate standard curves.

For quantitation of treponemal-, anti-CK-, and anti-Fn-specific subclass levels, two individual sera yielding high titres in each RIA were subjected to affinity chromatography for preparation of internal reference standards. *T. pallidum* soluble antigens, CK-RM and bovine Fn were coupled to separate lots of cyanogen-bromide-activated Sepharose CL-4B (Pharmacia, Piscataway, NJ) at a concentration of 1 mg/ml of packed Sepharose. Antibody was eluted with 3 M KSCN in PBS, pH 7·4, exhaustively dialysed and then concentrated to 10% of the original serum concentration using centrifugal microconcentrators (Amicon, Danvers, MA). Radial immunodiffusion kits (ICN ImmunoBiologicals) were then used to quantitate the IgG subclasses in these reference materials so that ELISA data could be expressed as μg/ml.

Statistical analysis

All data were stored in data bases, and statistical analyses performed with an EPISTAT statistical program (T. L. Gustafson, Round Rock, TX.) with the following exceptions. The Student's two-tailed *t*-test and non-parametric tests were performed using a computerized SAS Proc Test program.

RESULTS

Anti-VDRL responses

Using the optical density (OD) cutoff values (5 s.d. above the mean of the 15 normal controls) of 0·104 and 0·111 for the VDRL IgM- and IgG-ELISA, respectively, all of the sera from the patients with active secondary syphilis were positive in both assays. As shown in Fig. 1, IgM responses predominated in 17 of the 22 patients (62±21%). The mean percentages of the IgM and IgG in the six patients with primary syphilis were not significantly different ($P > 0·4$; data not shown). In contrast, IgG was the only class of anti-VDRL antibodies detected in four of the 11 treated patients, and in two others the average contribution of IgM was < 10% (data not shown).

Sera from the six secondary syphilis patients exhibiting the highest IgG-VDRL titres were further subjected to subclass analysis. As shown in Table 1, > 85% of the anti-VDRL IgG appeared attributable to IgG1 in both direct and indirect assays. Results obtained with the polyclonal reagent were in good agreement ($r > 0·951$) with those using the MoAb GG-5 and Pan-MoAb reagents. Results obtained in indirect assays employing protein A-affinity chromatography also were in agreement with restriction of the response to IgG1.

Anti-CK autoantibody responses

Using human and rabbit CK as capture antigens, sera were screened by RIA for IgG autoantibodies. As shown in Table 2, 16 of the 22 sera from patients with secondary syphilis exhibited reactivity with both antigens. By depletion analysis, 775 and 1100 cp/min in the MM-CK and RM-CK RIA, respectively, were estimated to represent 5 μg/ml of anti-CK-specific IgG. The mean IgG anti-CK responses in this patient population (MM-CK 1646±1073 ct/min; RM-CK 2398±1561 ct/min)

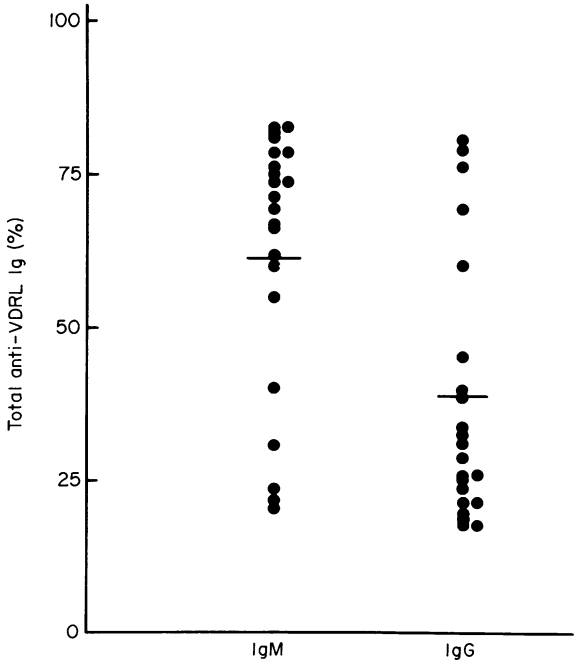

Fig. 1. Ig class of anti-VDRL antibodies in patients with secondary syphilis. Scaled percentages were based on the sum of IgM and IgG in μg/ml.

Table 1. Restriction of the VDRL-IgG Response to IgG1

Patient no.	Polyclonal IgG ELISA	*pan*-MoAb ELISA	IgG1-MoAb ELISA	
			Direct	Indirect
6	0·875±0·023*	0·723±0·018	0·650±0·031 (89·9)†	0·629±0·019 (87·0)
7	0·589±0·019	0·521±0·012	0·501±0·074 (96·2)	0·474±0·015 (91·0)
16	1·252±0·047	0·912±0·027	0·857±0·034 (94·0)	0·775±0·021 (85·0)
19	0·891±0·028	0·825±0·025	0·808±0·019 (97·9)	0·759±0·023 (92·0)
20	1·146±0·051	0·918±0·031	0·845±0·027 (92·0)	0·807±0·036 (87·9)
22	1·243±0·046	1·130±0·042	1·074±0·039 (95·0)	0·983±0·032 (87·0)

* Mean±s.d. of triplicate readings.

† Per cent of value obtained with *pan*-MoAb reagent.

Table 2. Anti-creatine kinase reactivity of secondary syphilitic sera by RIA and ELISA*

Patient	RIA (ct/min)		ELISA	
	MM-CK	RM-CK	IgG1 μg/ml	IgG3 μg/ml
1	3802	5064	12·1	8·8
2	2889	4039	6·6	7·8
3	1985	3131	6·0	4·1
4	380	490	<2·0	<2·0
5	2942	4898	14·3	3·8
6	2220	3335	7·2	5·8
7	276	445	<2·0	<2·0
8	315	414	<2·0	<2·0
9	1264	1889	4·1	2·9
10	3033	4990	8·6	9·8
11	2348	2396	5·5	4·9
12	226	356	<2·0	<2·0
13	1390	1997	2·1	4·6
14	1240	2043	2·6	5·2
15	1648	1605	4·9	2·3
16	2920	4220	5·3	12·2
17	315	414	<2·0	<2·0
18	2065	2990	8·9	2·8
19	234	377	<2·0	<2·0
20	1870	2834	5·2	3·9
21	1667	2750	5·6	4·4
22	1200	1650	3·7	2·9

* Radiolabelled polyclonal goat anti-human IgG was used in both RIA. For simplicity, all values represent the mean of triplicate determinations without s.d. which in all instances were less than 18%.

Table 3. IgG1 and IgG3 isotype restriction of anti-treponemal and anti-fibronectin antibodies in the sera of patients with secondary syphilis*

Patient	Anti-Tp IgG μg/ml	Anti-Tp IgG1 μg/ml	Anti-Tp IgG3 μg/ml	Anti-Fn IgG μg/ml	Anti-Fn IgG1 μg/ml	Anti-Fn IgG3 μg/ml
1	292	178	93	<2·0	<2·0	<2·0
2	126	56	46	23·9	15·5	7·9
3	141	80	38	17·4	9·2	8·5
4	157	56	64	<2·0	<2·0	<2·0
5	64	26	32	27·6	13·5	11·8
6	61	18	40	32·9	19·7	9·9
7	206	128	86	9·7	5·6	5·1
8	203	72	102	6·3	2·3	3·7
9	284	216	82	12·2	3·7	7·3
10	147	76	40	13·7	4·2	9·0
11	318	151	126	3·4	<2·0	<2·0
12	117	28	84	17·3	8·9	6·1
13	53	22	26	25·3	9·6	13·2
14	382	148	169	7·6	3·9	2·9
15	318	185	117	<2·0	<2·0	<2·0
16	363	119	204	2·9	<2·0	<2·0
17	53	21	37	24·4	15·1	10·1
18	44	24	14	<2·0	<2·0	<2·0
19	428	258	153	<2·0	<2·0	<2·0
20	285	115	136	5·9	2·6	2·5
21	148	83	52	25·8	12·3	11·4
22	377	131	223	<2·0	<2·0	<2·0

*Total IgG responses were determined using the *pan*-MoAB reagent. For simplicity, all values represent the mean of triplicate determinations without s.d. which in all instances were less than 18%.

were significantly greater ($P < 0{\cdot}0001$) than the means of those of the normal controls (MM-CK 178 ± 64; RM-CK 203 ± 56), patients with primary (MM-CK 228 ± 103; RM-CK 341 ± 197) or treated syphilis (MM-CK 541 ± 408; RM-CK 632 ± 486). IgM anti-CK activity was absent in all sera (data not shown).

Using RM-CK-affinity-purified preparations from patients no. 1 and no. 10 as internal reference standards, attempts were made to determine which isotypes were contributing to these responses. The IgG1 and IgG3 results obtained in subclass ELISA using RM-CK as the antigen also are shown in Fig. 2. While none of the sera contained a single anti-CK isotype, dramatic skewing to these two subclasses was noted with IgG3 being the predominant subclass in five patients. The average contribution of IgG1 was 49 ± 14% with IgG3 contributing slightly less: 44 ± 16%. As neither reference standard contained detectable IgG2 and IgG4 anti-CK antibodies, this approach was not feasible for assessing the contribution of these isotypes. The relative contribution of IgG2 and IG4, estimated by comparing subclass-specific optical densities with those using the *pan*-MoAb, suggested that these subclasses accounted for less than 8% and 5% of the total activity, respectively, in all but two patients. In those patients, using this approach, IgG2 accounted for approximately 20 and 15% of the total response. IgG1 was the only measurable response in the one patient with primary syphilis and the four treated patients with sufficiently elevated anti-CK activity (data not shown). No associations appeared to exist between the anti-CK subclass responses and anti-*T. pallidum*-specific subclass responses using Pearson's correlation coefficient test.

Anti-Fn autoantibody responses

Similar methodologies, employing CBF of bovine and human Fn, were used to evaluate class and subclass responses to Fn. Sixteen of the sera from patients with secondary syphilis were found to contain elevated IgG levels of anti-Fn activity in RIA. None of the sera from the other three patient groups were positive. Additionally, IgM anti-Fn antibodies were not detected in any sera.

Table 3 shows the IgG1 and IgG3 results obtained by ELISA using CBF of bovine Fn as the antigen and affinity purified materials from patients no. 2 and no. 6 as internal reference standards. Affinity-purified materials from patients no. 14 and no. 18 served as the internal standards in the anti-*T. pallidum*-specific ELISA and the total IgG responses were determined using the *pan*-MoAb reagent in both instances. Only 14 of 16 patients with elevated antibody to Fn in the RIA had sufficient activity for IgG subclass determinations. Antibodies to Fn distributed as 49 ± 12% IgG1 and 44 ± 13% IgG3. Subclass activity to the CBF of human Fn paralleled the results obtained using CBF of bovine Fn (data not shown). The relative contribution of IgG2 and IgG4, again based on OD comparisons with those using the *pan*-MoAb, indicated that these subclasses accounted for less than 12 and 5%, respectively, in all instances. A strong inverse correlation ($r = -0{\cdot}873$, $T = 6{\cdot}69$

and $P < 0{\cdot}00001$) was found between the total anti-treponemal IgG and total anti-Fn IgG responses using Pearson's correlation coefficient test (columns 2 and 5, Table 3; $n = 16$ patients). Similarly, an inverse correlation ($r = -0{\cdot}896$, $T = 6{\cdot}97$, $P < 0{\cdot}00002$; $n = 14$) also was evident between the anti-treponemal and anti-Fn IgG3 responses.

Autoantibody responses to other host proteins

Similarly contructed RIA and ELISA employing other capture antigens were used to screen sera for autoantibodies to other host proteins. Five sera from patients with secondary syphilis contained elevated IgG antibodies to type IV collagen, while three others had elevated anti-laminin IgG levels. The only recognizable subclass responses to laminin and to type IV collagen when these sera were retested by ELISA were IgG1 (data not shown). IgM reactions with these proteins were not detected. All sera were negative in RIA employing polymerized HSA for the detection of anti-albumin autoantibodies.

DISCUSSION

Secondary syphilis is characterized by co-ordinate, restricted expression of IgG1 and IgG3 responses to antigens of *T. pallidum* (Baughn *et al.*, 1988); IgG3 activity has been considered disproportionate based on the low levels of the isotype in sera. In the present study, we attempted to characterize the class and subclass distribution of several autoantibodies. In agreement with earlier studies, both in our laboratory and by other investigators (Casavant *et al.*, 1978; Fitzerald *et al.*, 1984; Strugnell *et al.*, 1986; Baughn *et al.*, 1986, 1987; Baughn 1987), we found autoimmune responses in active syphilis directed primarily against VDRL antigen, CK and Fn. Our data show that the predominant responses to CK and Fn occurred in IgG1 and IgG3 subclasses, while activity against the VDRL antigen was exclusively IgG1.

The restricted isotype distribution seen in the anti-CK and anti-Fn experiments, while impressive, are not unique; several studies have suggested that autoantibody responses are restricted to these two subclasses (Schur, Monroe & Rothfield, 1972; Puritz, *et al.*, 1973; Sontheimer & Gilliam, 1978; Yount, Dyer & Fuller, 1983; Zouali, Jefferis & Eyquem, 1984; Eisenberg *et al.*, 1985; Rubin *et al.*, 1986). In systemic lupus erythematosus in particular, anti-DNA and anti-nuclear antibodies have been shown to be predominantly IgG1 and IgG3 responses. Restriction of anti-CK and anti-Fn responses to these isotypes, like those to *T. pallidum*, could reflect basic functional and/or mechanistic linkages, since two of the most plausible explanations include immaturity of the immune response or polyclonal activation mediated by a common subset of subclass-specific T cells (Rubin *et al.*, 1986).

While the relative proportions of anti-CK and anti-Fn subclass responses seemingly paralleled anti-treponemal activity, much of the present evidence argues against associations between autoimmune and anti-*T. pallidum* subclass responses. The exception is the inverse relationship between anti-Fn IgG3 and anti-treponemal IgG3. It is tempting to postulate that Fn, which unlike CK and cardiolipin (as in the VDRL antigen), has a high binding affinity for *T. pallidum* (Peterson, Baseman & Alderete, 1983; Baughn, 1986), may be presented differently from a normally sequestered antigen which might be freed only after tissue damage. Although this inverse relationship would be consistent with our recent hypothesis that anti-Fn activity actually is a reflection of anti-idiotypic responses to antibodies against the 83-kD Fn-binding protein (Baughn, in press), these data and the resulting association must be viewed cautiously. They could merely represent a co-linked variable without pathogenetic implications.

In the case of antibody to VDRL, IgM activity predominated with nearly all of the detectable IgG confined to IgG1. While our results are in agreement with earlier studies (Gharavi *et al.*, 1986; Pedersen *et al.*, 1987; Harris *et al.*, 1988) in regard to differing profiles of reactivity with syphilitic sera, they do not appear to be linked either to the specific antitreponemal responses or to the other autoimmune responses. This raises the intriguing question as to whether multiple mechanisms are involved in the evolution of these various responses. The lack of association between the VDRL-IgM and total anti-treponemal IgM responses may additionally constitute evidence against generalized polyclonal activation of B cells in syphilis resulting in the non-specific production of autoantibodies. Clearly, much additional work is necessary for a better understanding of the origin, maintenance, subsequent suppression, and pathogenesis of autoimmune responses in syphilis.

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